THE EFFECT OF ETHYL CARBAMATE ON SUCCINATE DEHYDROGENASE ACTIVITY

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CHAPTER I

INTRODUCTION

The question of the effect of ethyl carbamate on living cells and, especially, on the cell parts has acquired considerable attention. Ethyl carbamate (urethane) belongs to a homologous series of carbamates differing in the alkyl chain length. The chemical formula of ethyl carbamate is $NH_2COOC_2H_5$.

Ethyl carbamate is water soluble, therefore, it may penetrate cells more readily than the homologs with longer chains, which become less soluble as the number of carbon atoms increases. Ethyl carbamate is an anesthetic which has been used on laboratory animals. Anesthetics are known to be metabolic antagonists.

Ethyl carbamate has also been found to act on isolated living tissue or on the whole organism, which may be indicative of its interference with metabolism by acting as an inhibitor. The products which result from the metabolic alterations by inhibitors may be either active or inactive.

In view of the often inconsistent, although sometimes selective effects of ethyl carbamate, this study was designed to determine its action on the time relationship of the inhibition in vivo; this included the rate at which the inhibition was developed, and the degree of reversibility of the inhibition.

CHAPTER II

REVIEW OF LITERATURE

Urethane (ethyl carbamate) was first synthesized by Dumes in 1834 from ethylchloroform and ammonia (1). It was the first of the drugs related to urea to be used as a somnifacient. Since the discovery of ethyl carbamate its action on various tissues has been under observation.

In 1943, Nettleship, Henshaw, and Meyer (2) reported multiple pulmonary tumors in experimental C_3H mice exposed to X-rays. After some probing and studying they found that the causative agent was the anesthetic, urethane, which led to further studies of this chemical agent. Ethyl carbamate was reported to be a multipotential carcinogen in mice (3). According to Kawamoto, Kirschbaum, and Taylor (4), ethyl carbamate augmented the induction of leukemia in mice by X-rays, estrogenic hormone, or methylcholanthrene. Berenblum and Haran-Ghera (5) administered large doses (120-60 mg.) of ethyl carbamate to swiss mice by stomach tube. Their studies demonstrated the development of multiple papillomas of the squamous epithelium of the forestomach with ethyl carbamate treatment. In C_3H strains of mice, which have been demonstrated to produce spontaneous hepatomas, the incidence of hepatomas was less in C_3H males treated with ethyl carbamate and greater in similarly treated C_3H females (6). These animals were castrated before treatment. It was concluded from the findings that the hormonal environment of the host influenced the development of tumors.

Ethyl carbamate was reported to inhibit the growth of the Walker tumor (7). Boyland and Koller (8) demonstrated that ethyl carbamate induced mitotic injuries in the Walker carcinoma 256. The effect of ethyl carbamate was

reduced in the Walker tumors by treatment with thymine. Boyland and Koller's (8) findings suggested that ethyl carbamate affected chromosome synthesis indirectly and that the damage in the rat was similar to that produced by irradiation. They proposed that ethyl carbamate interfered with thymine synthesis which would cause a deficiency of thymidine or deoxyribonucleic acid. In Lanitzki's (9) studies on the of urethane on growth and mitosis of normal and malignant cells in vitro, cultures obtained from normal tissue indicated a reduction of growth and cultures of malignant cells, a stimulation of growth. Investigations by Bastrup-Madsen (10) demonstrated that ethyl carbamate had a general cytotoxic effect, and that in certain concentrations (0.66-1.2%) the dividing cell was damaged. His findings also suggested that mitotic activity was inhibited at metaphase. Ethyl carbamate has also been used in the treatment of leukemia in man (11).

Van Breeman (12) treated onion root tips with the following series of aqeous ethyl carbamate solutions: 0.1M, 0.2M, and 0.3M. Changes in length of the roots were measured before, during, and after treatment. He found that treatment with ethyl carbamate caused a slowing down and eventual cessation of growth of the onion root which was proportional to the concentration used.

Early contributions of Warburg (13) indicated that a series of carbamates, with regards to chain length, formed a progression of narcotic effectiveness when tested on sea urchin eggs, and that cell division was disrupted at concentrations that barely affected oxygen consumption. On the contrary, Bodine and Fitzgerald (14) found that a series of carbamates reduced the oxygen consumption in grasshoppers. The effect of a series of concentrations of ethyl carbamate were determined on the consumption of oxygen by fertilized and unfertilized eggs of the sea urchin (15). It was found from the relationship of concentration to inhibition that there were two parallel respiratory systems

affected by ethyl carbamate and when one of these was inactivated cell division was blocked (15).

Examination of a homologous series of carbamates for their effect on bacterial luminescence demonstrated the relationship of temperature changes as the alkyl chain length increased (16). Ethyl carbamate was reported as acting in a manner that promoted the breaking of the type of bonds broken in both the reversible and irreversible enzyme reactions and promoting the irreversible denaturation.

A tentative sequence was proposed as to the site of inhibition by narcotics. This was demonstrated by adding ethyl carbamate to a yeast suspension and shaking with oxygen which upon spectroscopic examination demonstrated the oxidation of the cytochromes. Upon the addition of cyanide, the cytochromes did not become reduced. Later, Sen (17) demonstrated that narcotics inhibited the activity of dehydrogenases and, therefore, the reduction of oxidized cytochromes. This was done by adding ethyl urethane to enzyme preparations and measuring the reduction.

Results from the investigation mentioned above indicated that ethyl carbamate is not always consistent in its action on the cell, however, under a given set of conditions it may be selective. Ethyl carbamate has been reported to act as a narcotic, carcinogen, carcinoclast, and a mitotic poison. According to Warburg (13), a change in cell respiration was the determining factor in causing malignancy or tumors. Assuming that this theory is acceptable, ethyl carbamate may act as a carcinogen and a carcinoclast based on the findings that it also acts as an inhibitor of respiration and this may indicate or suggest that the controlling factor is concentration. According to Cornan (18), when ethyl carbamate acted as a narcotic it affected cell division by depriving the process of oxidation energy. Therefore, the four

modes of action of ethyl carbamate appeared to be dependent on the degree of inhibitory effects it had upon the cell.

Succinate dehydrogenase is an enzyme of the Kreb's cycle and has been reported as being firmly attached to the mitochondria. This enzyme contains an apoenzyme and a co-enzyme, flavo-protein (19). According to Bonner (20), there has been no successful method for isolating succinate dehydrogenase from cellular inclusions. Therefore, assays for this enzyme were carried out in the presence of the succinate dehydrogenase system which included some or all of the components of the cytochrome system. Succinate dehydrogenase has been reported as the only enzyme of the Kreb's cycle to contain a flavo-protein to date; hence, it was the only enzyme of the Kreb's cycle that was part of the electron transport system (19, 20, and 21). Tsou (21) has presented evidence which indicated that succinate dehydrogenase was the immediate electron acceptor from succinate. Stoppani (22) used two of the carbamates, phenylurethane and ethylurethane, in his studies on the mechanism of succinoxidase inhibition. Stoppani's investigation demonstrated a linear relationship between the intensity of the inhibition and the concentration of phenylurethane using liver as a source of succinoxidase. He also used several dyes to test whether or not the redox potential of the hydrogen acceptor would influence the intensity of the inhibition and found no affect. Stoppani concluded that the two carbamates used inhibited the mechanism that transferred hydrogen from succino-dehydrogenase either to dyes or to cytochrome c but not the reduction of ferricyanide by succinate. The inhibition by phenylurethane was in concentrations that brought about narcosis. Tsou (21) explained the findings of Stoppani (22) by assuming that ferricyanide was capable of reacting directly with succinate dehydrogenase, whereas methylene blue can only react with cytochrome b and that narcotics affected the inter-

action of the dehydrogenase and cytochrome b.

CHAPTER III

MATERIALS AND METHODS

The C_3H mice used in this experiment were originally obtained from Rockland Farms, New City, New York and Gilbertsville, Pennsylvania. All animals were housed in wire cages and fed Burger bits ad libitum. The animals were separated into two group (test and control). The test animals were injected intraperitoneally (1 mg. per gm. body weight) with ethyl carbamate dissolved in physiological saline (0.9%). The control animals were injected intraperitoneally with a corresponding volume of the saline solution.

Animals were sacrificed by a firm blow on the head, decapitated, and bled at intervals of 6, 12, 24, 48, 72, 96 and 144 hours after injection. The procedure for enzyme preparation was followed as suggested by Bonner (20) with a few modifications. The liver was removed immediately and placed in cracked ice; after cooling, the liver was washed in cold distilled water, blotted dry and weighed. The liver was minced, rewashed in cold distilled water and centrifuged to remove water soluble substances. The minced liver was homogenized at $0^{\circ}C$ with 10 volumes of phosphate buffer (0.1M pH 7.2) which served as an extracting medium. The homogenate was centrifuged at 1500 x G for 20 minutes at $0^{\circ}C$ and the cloudy supernatant was removed. The supernate was rewashed with phosphate buffer (0.1M pH 7.2) and centrifuged at 1500 x G for 20 minutes at $0^{\circ}C$. The supernatant from the first and second washing were pooled and subjected to further centrifugation at 10,000 x G for 20 minutes at $0^{\circ}C$. The precipitate was collected and suspended in an equal volume of the phosphate buffer.

Samples of the enzyme preparation, urine, and plasma were chromatographed

for detection of ethyl carbamate. The solvent system consisted of butanol-acetic acid-water (12: 3:5 by vol). The chromatgrams were dried in an oven at 45°C from 2-10 minutes. The chromatograms were sprayed with 0.05% (w/v) nindydrin in acetone and followed by heating at 90°C for 5 minutes. An ultra-violet light was used for locating spots which were not stained by ninhydrin.

The Thunberg method which dependsupon the rate of reduction of a suitable hydrogen acceptor under anaerobic condition was employed for measuring succinate dehydrogenase activity (23). The following components were pipetted into the Thunberg tubes:

TUBES	THUNBERG TUBES				THUNBERG STOPPER HOMOGENATE	
	H_2O ml.	BUFFER ml.	SUCCINATE ml.	METHYLENE BLUE ml.	URETHANE ml.	SALINE ml.
1	0.0	4.0	1.0	1.0	0.5	0.0
2	0.0	4.0	1.0	1.0	0.5	0.0
3	1.0	4.0	0.0	1.0	0.5	0.0
4	0.0	4.0	1.0	1.0	0.0	0.5
5	0.0	4.0	1.0	1.0	0.0	0.5
6	1.0	4.0	0.0	1.0	0.0	0.5

Once the enzyme preparation was placed in the side cap, stopcock grease was applied to the joint of the cap and firmly placed on the tubes. Ten minutes of evacuation was carried out with a saturated solution of pyrogallic acid in 70% KOH and a water aspirator so that methylene blue could not be oxidized by air after it was reduced to its colorless form. The tubes were allowed to come to equilibrum for 10 minutes in a 37°C water bath; after this the contents of the tubes were mixed and the reduction of methylene blue was measured

photometrically (Klett-Summerson) using a red filter. The tubes were read immediately after mixing the contents, returned to the water bath, and read every 3 minutes thereafter. For complete reduction, a few crystals of Na_2SO_4 were added after 30 minutes. The tubes were read and the concentration of methylene blue was calculated for the time period measured before complete reduction. A unit of enzyme activity was that amount of enzyme which produced 1 µg. of colorless methylene blue in 12 minutes at 37°C.

CHAPTER IV

EXPERIMENTAL RESULTS AND DISCUSSION

Various concentrations of ethyl carbamate were intraperitoneally injected into C_3H male and female mice. As the concentration increased, the anesthetic effectiveness increased. All animals which received more than 1 mg. per gm. body weight became sluggish 5 minutes after intraperitoneal injection of ethyl carbamate, and the duration of anesthetization was proportional to the amount received. Animals which received less than 1 mg. per gm. body weight became active and began to eat 5 minutes after intraperitoneal injections.

Ethyl carbamate inhibited the activity of succinate dehydrogenase in C_3H mice liver at doses below the anesthetic level. The succinate dehydrogenase activity of the control animals which received an equivalent volume of 0.9% saline was not supressed. Six hours after injection in the control animals the conversion of methylene blue to the leuco-methylene blue was much greater than in the experimental animals as shown in Figure 1.

The decolorization of the dye was measured and recorded at 3 minute intervals. The succinate dehydrogenase activity was that unit which equalled 1 µg. per mg. of liver methylene blue which was converted to leuco-methylene blue in 12 minutes. The conversion of methylene blue can be seen in the illustration below:

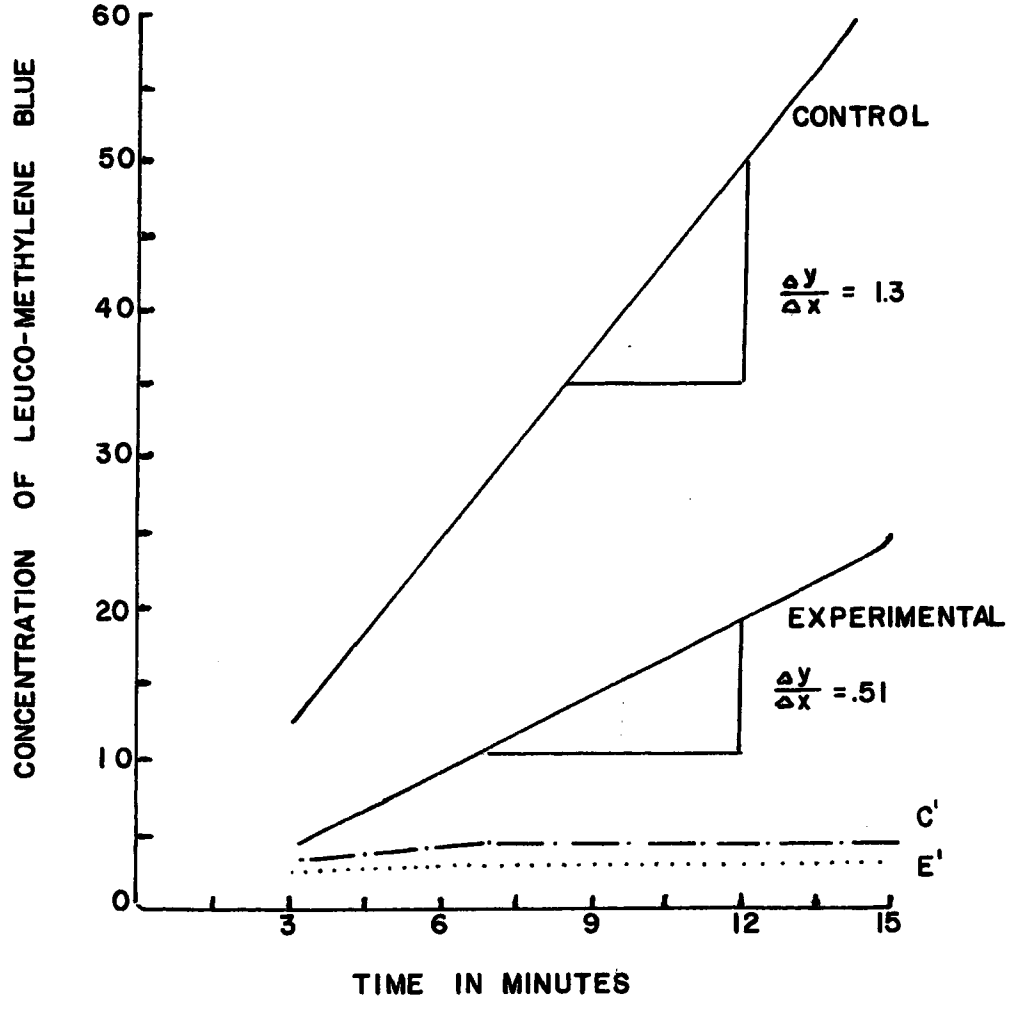
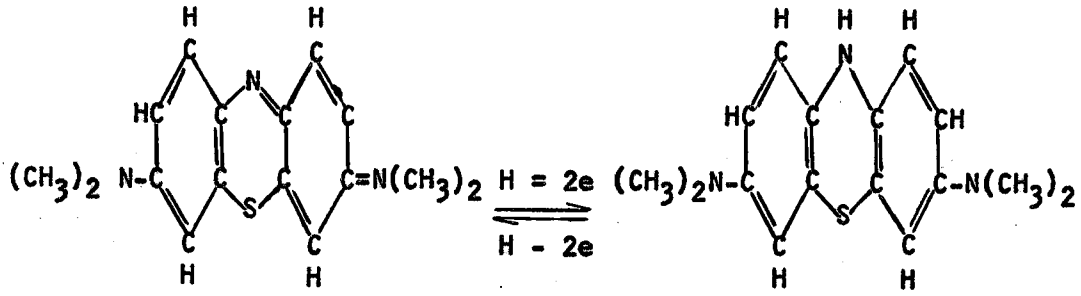


Fig. 1. Inhibition of anaerobic succinate oxidation 6 hours after injection. The ordinate represents concentration of leucomethylene blue (1×10^{-3} µg.). E' = the endogenous enzyme activity of the experimental system, while C' = the endogenous enzyme activity of the control system.



There was a linear relationship between the activity of succinate dehydrogenase and time in both the control and experimental systems. The average slope of the control plots was 1.3-1.5, whereas, the slope of the experimental systems was 0.51 6 hours after injection. In 12 minutes 19.5×10^{-3} µg. of leuco-methylene blue were present in the experimental system; whereas, 50.0×10^{-3} µg. of leuco-methylene blue were present in the control systems. A control of each enzyme preparation was run without substrate in the system in order to determine the endogenous enzyme activity. The endogenous enzyme activity was completed by 9 minutes in each system which contained no substrate. Subtracting the endogenous values from the experimental and control measurements, wherein substrate was present, increased the activity by 41.3 times in the absence of the inhibitor and by 15.0 when the inhibitor was present. Thus, the inhibition due to ethyl carbamate was actually 63.6% when the two values were compared.

The units measured 12 hours after injection were higher than those of 6 hours giving a slope of 0.91 as shown in Figure 2. The activity was 36.0×10^{-3} units 12 hours after injection.

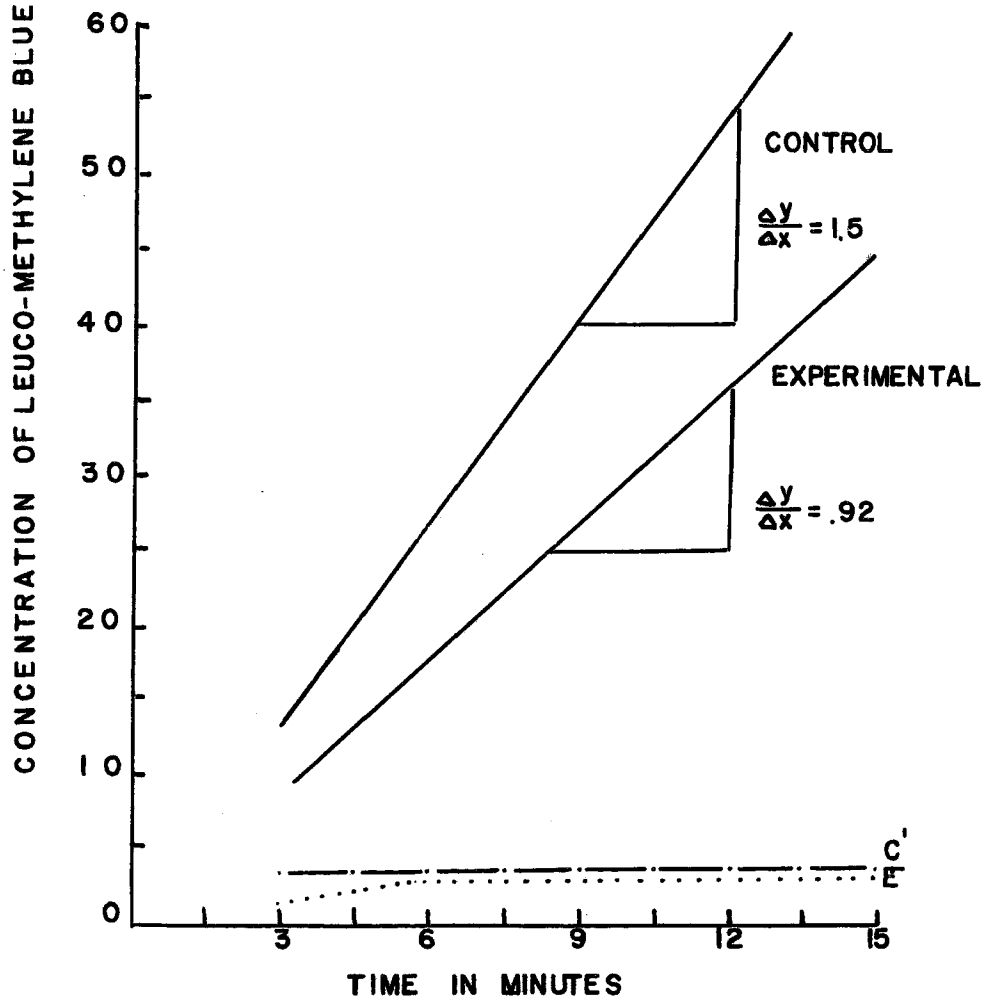


Fig. 2. Anaerobic succinate oxidation 12 hours after injection. The concentration of leuco-methylene blue was 1 X 10^{-3} µg. E' = endogenous enzyme activity of experimental systems. C' = endogenous enzyme activity of control systems.

The addition of substrate increased the metabolism by a factor of 51.4 in the absence of the inhibitor and 33.0 when the inhibitor was present; hence, the inhibition of substrate succinate dehydrogenase was 35.8%.

In 24 hours the units determined were less than those obtained at 12 hours (Fig. 3). The slope of the plot was 0.69. It was thought that the results obtained 12 hours after injection were due to food intake before the animals were sacrificed; therefore, the animals sacrificed at 6, 12, 24 hours after injection were fasted for 24 hours. The results obtained were the same. The inhibition of substrate activity was 55%.

The slope of graphic results obtained 48 hours after injection was 1.0 (Fig. 4) demonstrating that the activity of succinate dehydrogenase was still inhibited. The inhibition of the substrate enzyme was 23%.

The slope at 72 hours after injection was 1.01 (Fig. 5), and at 96 and 144 hours (Figs. 6 and 7 respectively) after injection the slope of the plots had reached the range of the controls. The inhibition of substrate succinate dehydrogenase 72 hours after injection was 8.4% when corrected for endogenous enzymatic activity and compared with the controls.

Figure 8 shows the inhibition and rate of inhibition with regards to time after injection of ethyl carbamate. The units are plotted against time in hours.

The inhibition of succinate dehydrogenase may have an important role in the narcotic action of ethyl carbamate.

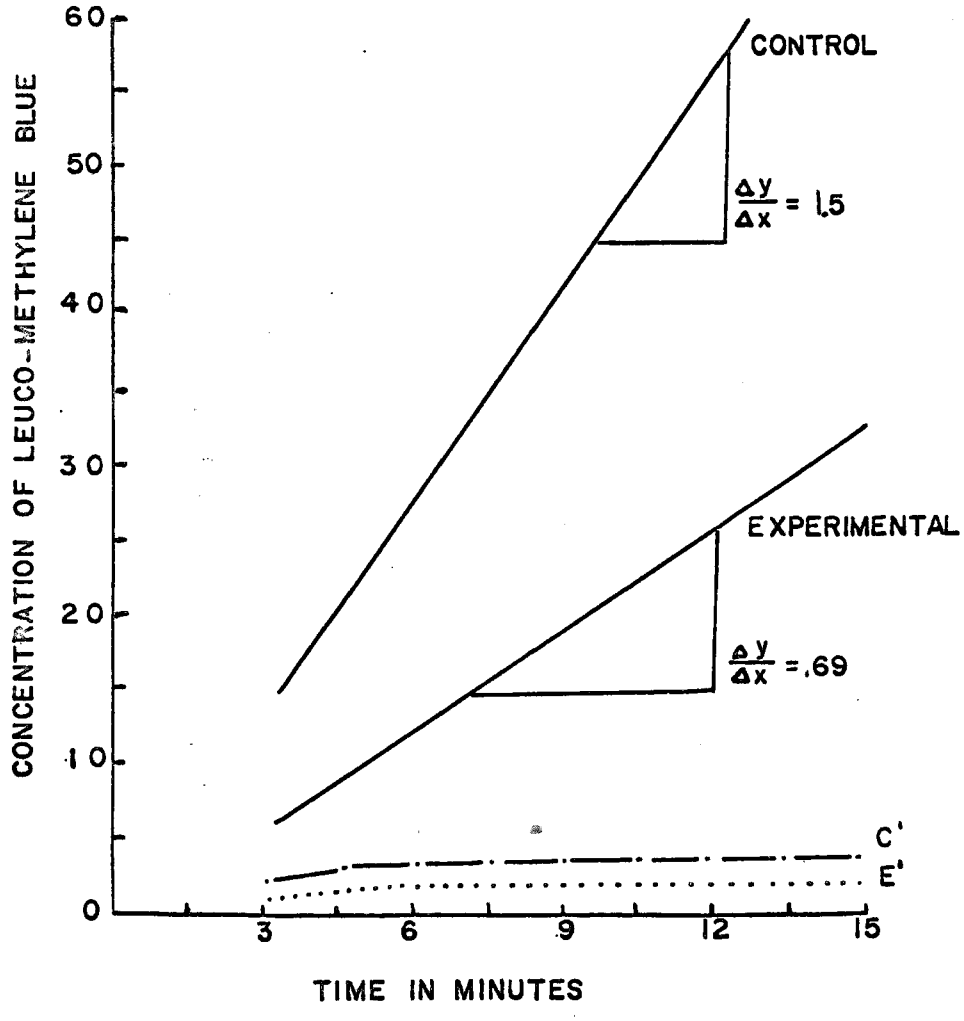


Fig. 3. Anaerobic succinate oxidation 24 hours after injection. The concentration of leuco-methylene blue was 1 X 10^{-3} µg. E' = endogenous enzyme activity of experimental systems. C' = endogenous enzyme activity of control systems.

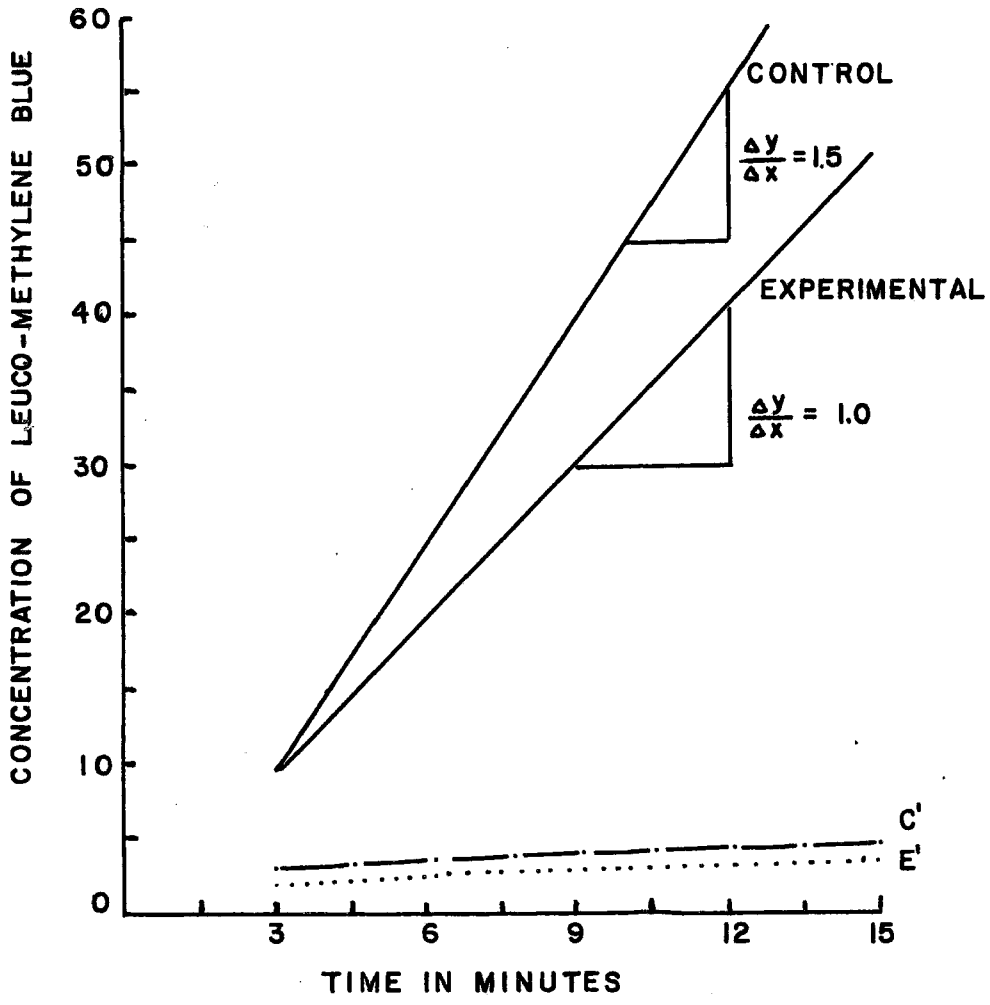


Fig. 4. Anaerobic succinate oxidation 48 hours after injection. The concentration of leuco-methylene blue was 1 X 10^{-3} µg. E' = endogenous enzyme activity of experimental systems. C' = endogenous enzyme activity of control systems.

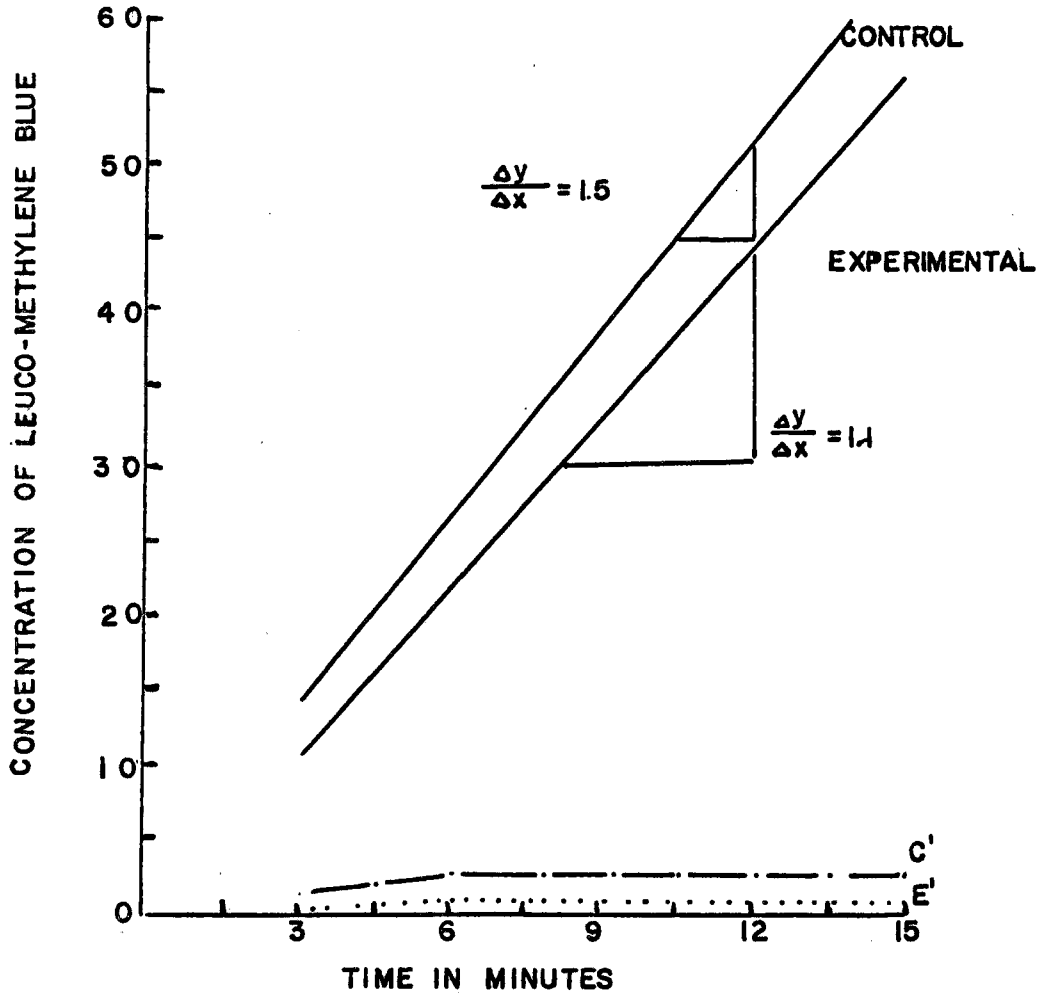


Fig. 5. Anaerobic succinate oxidation 72 hours after injection. The concentration of leuco-methylene blue was 1 X 10^{-3} µg. E' = endogenous enzyme activity of experimental systems. C' = endogenous enzyme activity of control systems.

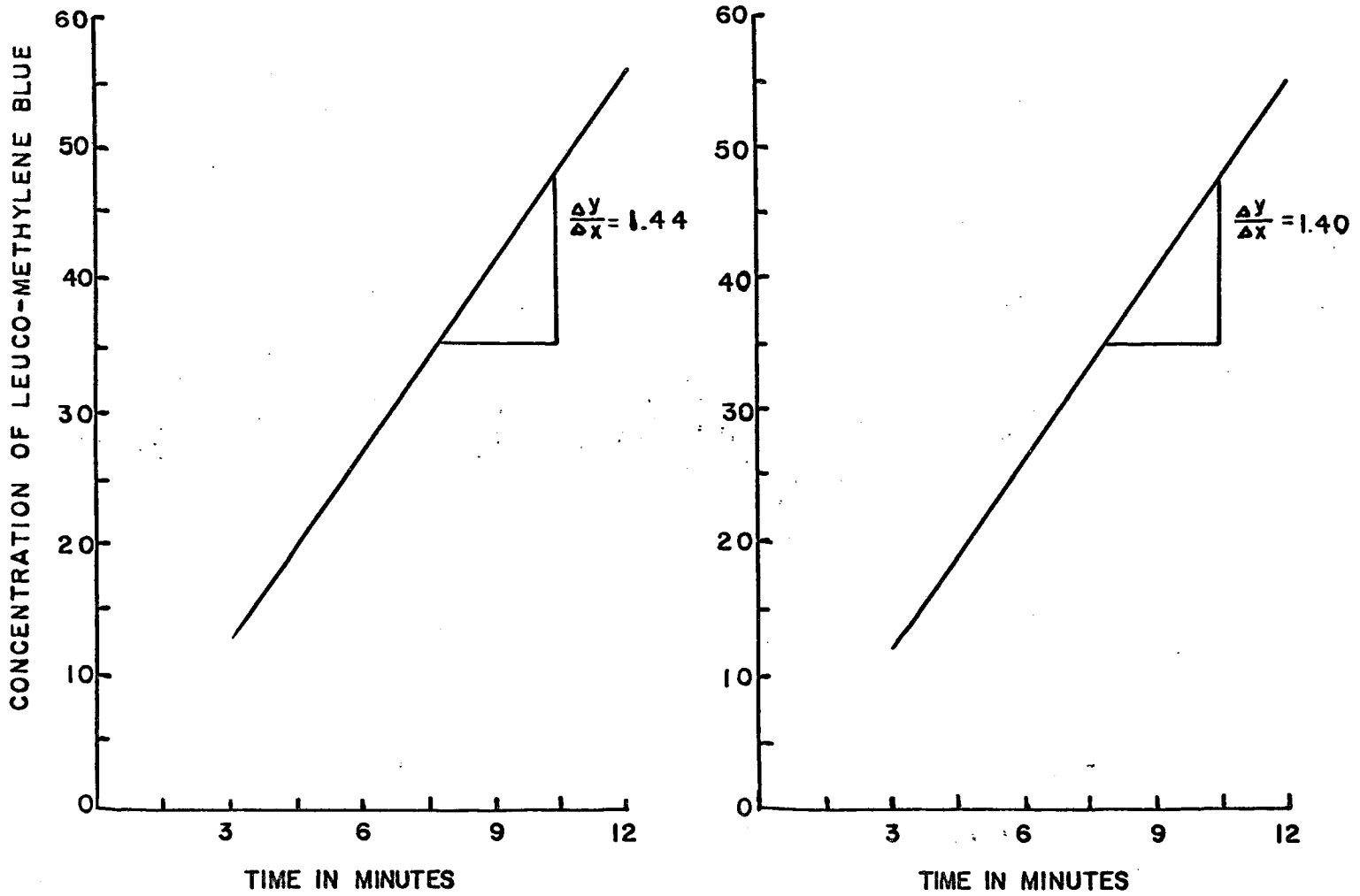


Fig. 6. Graph from data of experimental animals on anaerobic succinate oxidation 96 hours after injection. The concentration of leuco-methylene blue was 1 X 10^{-3} µg.

Fig. 7. Graph from data of experimental animals on anaerobic succinate oxidation 144 hours after injection. The concentration of leuco-methylene blue was 1 X 10^{-3} µg.

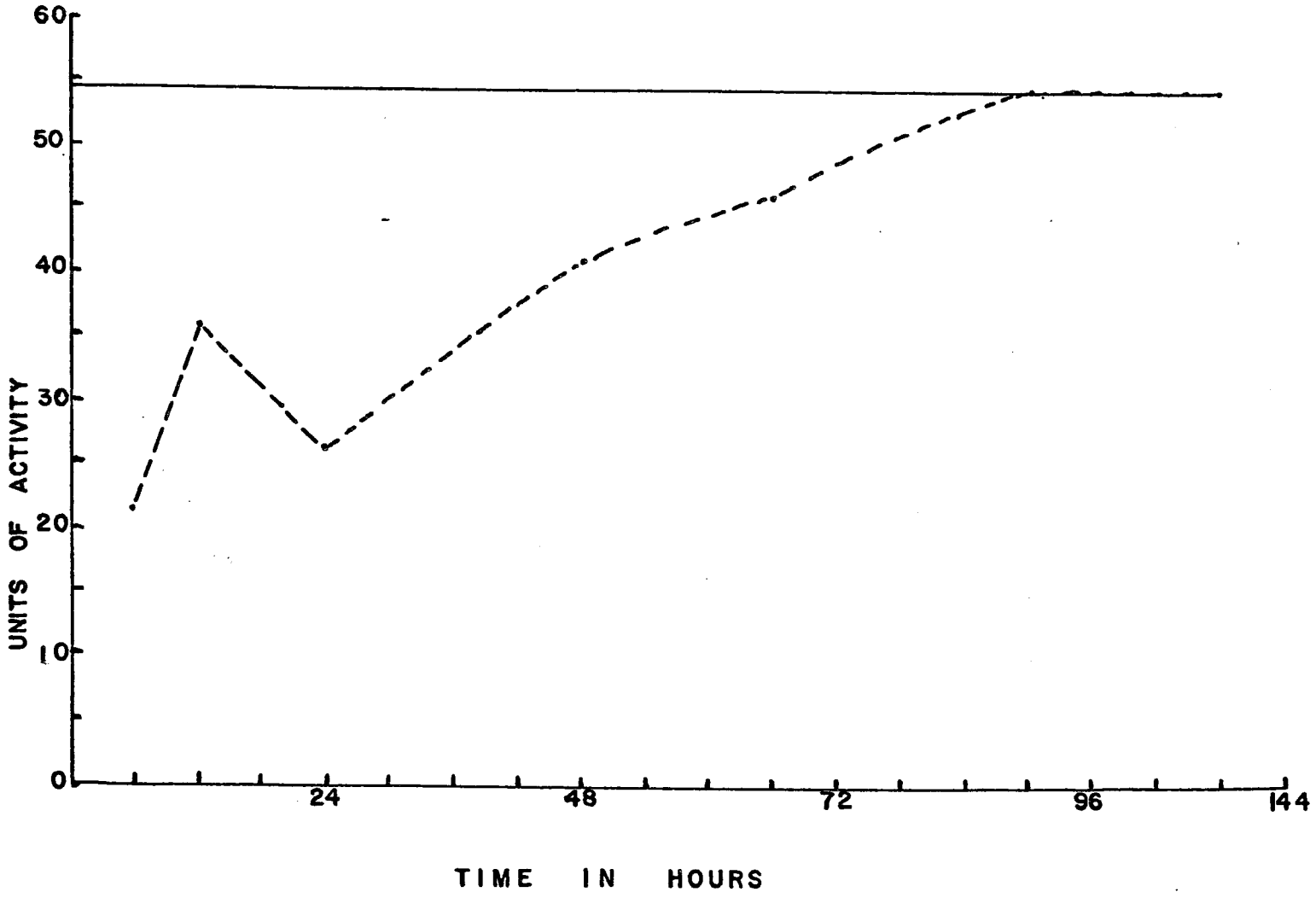


Fig. 8. Summary of succinate dehydrogenase activity after injection. The units of activity are 1 µg per mg. of liver per 12 minutes. Time after injection is plotted on the abscissa. The solid lines represents the average of control values; the dotted lines represents the average of the experimental

It has been demonstrated that ethyl carbamate inhibited succinate dehydrogenase activity; thus, it may influence a particular functional activity of cells and/or organs.

Narcotic doses of phenylurethane and ethylurethane *in vitro* have been demonstrated to inhibit 40-60% of succinate dehydrogenase activity with methylene blue under anaerobic conditions (22). Doses of ethyl carbamate below the anesthetic level used in this experiment inhibited succinate dehydrogenase activity by 64% of its normal value. This dosage may be high enough to inhibit succinate dehydrogenase activity completely when isolated from the cell; however, a possible explanation of partial inhibition of the intracellular enzyme may be the failure of the inhibitor to enter the cell at the external level or the environment of the intracellular enzyme is different.

The difficulty in obtaining a linear relationship between inhibition and rate of inhibition may be due to a latent period and/or some metabolic response. It could be assumed that the units obtained at 12 hours after injection were due to synthesis of succinate dehydrogenase to compensate for the inactivation of the succinate dehydrogenase that was present. The rise in the slope could also be due to an increase in the metabolism of glucose; however, fasting the animals 24 hours before sacrificing did not rule out the possibility of other metabolic alterations. Once animals were fasted it is possible that they began to metabolize what fat was present. It can be seen that by 96 hours the experimental values have approximated those of the controls, and this may be interpreted as either total destruction or elimination of ethyl carbamate, or increased synthesis of succinate dehydrogenase.

Repeated doses every 3 days clearly demonstrates a way in which concentration of the inhibitor in the tissue may be built up to high levels which could possibly have an additive effect with regards to inhibition on the total

organism. The inhibitory action of ethyl carbamate on succinate dehydrogenase activity does not necessarily imply that it is specific for succinate dehydrogenase. Urethanes have been reported as being specific for the dehydrogenases (20). Johnson et al. (16) suggested that urethanes in Photobacterium act in a manner that promotes a reversible reaction which is coupled with promoting the irreversible protein denaturation. Accepting this proposition, it can be assumed that all enzymes similar in structure to the affected enzyme in Photobacterium may be affected by ethyl carbamate.

The inhibitory action of ethyl carbamate on mitosis (8, 9, 12, and 18) may be understood much more clearly if it is assumed that oxidative energy is needed for respiration, and succinate dehydrogenase is involved in the transfer of electrons (19). The concentration of ethyl carbamate appears to be the leading factor involved; thus, narcotic doses (17) may affect cell division and thereby act as a mitotic poison and a carcinoclast. Narcotic doses of urethane have also been demonstrated to inhibit luminescence in Photobacterium (16). It has been found that narcotic doses of the longer chain homologs of urethane have inhibited succinate dehydrogenase activity, whereas, in this study doses below the narcotic level of ethyl carbamate have inhibited succinate dehydrogenase activity.

CHAPTER V

SUMMARY AND CONCLUSIONS

C_3H male and female mice were injected intraperitoneally with increasing doses of ethyl carbamate to determine the anesthetic dosage. One mg. per gm. body weight of ethyl carbamate did not anesthetize the animals.

One mg. per gm. body weight of ethyl carbamate, which is below the anesthetic dosage was injected intraperitoneally into C_3H male and female mice to determine the effect on succinate dehydrogenase activity. Ethyl carbamate inhibited anaerobic succinate oxidation with methylene blue as a carrier. The endogenous enzyme activity was measured for each enzyme preparation. The percentage of inhibition was that value minus the endogenous enzyme activity. Six hours after injection of ethyl carbamate 63% inhibition was noted of anaerobic succinate oxidation.

The inhibition decreased with time; hence, by 4 days anaerobic succinate oxidation was within the control range.

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